

# Systemic Delivery of Human Mesenchymal Stromal Cells Combined with IGF-1 Enhances Muscle Functional Recovery in LAMA2<sup>dy/2j</sup> Dystrophic Mice

Mariane Secco · Carlos Bueno Jr · Natassia M. Vieira · Camila Almeida · Mayra Pelatti · Eder Zucconi · Paolo Bartolini · Mariz Vainzof · Elen H. Miyabara · Oswaldo K. Okamoto · Mayana Zatz

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**Abstract** The combination of cell therapy with growth factors could be a useful approach to treat progressive muscular dystrophies. Here, we demonstrate, for the first time, that IGF-1 considerably enhances the myogenesis of human umbilical cord (UC) mesenchymal stromal cells (MSCs) in vitro and that IGF-1 enhances interaction and restoration of dystrophin expression in co-cultures of MSCs and muscle cells from Duchenne patients. In vivo studies showed that human MSCs were able to reach the skeletal muscle of LAMA2<sup>dy/2j</sup> dystrophic mice, through systemic delivery, without immunosuppression. Moreover, we showed, for the first time, that IGF-1 injected systemically together with MSCs markedly reduced muscle inflammation and fibrosis, and significantly improved muscle strength in dystrophic mice. Our results suggest that a combined treatment with IGF-1 and MSCs enhances efficiency of muscle repair and, therefore, should be further considered as a potential therapeutic approach in muscular dystrophies.

**Keywords** Muscular dystrophies · IGF-1 · Mesenchymal stromal cells

## Introduction

Progressive muscular dystrophies (PMDs) are a group of disorders characterized by gradual muscle degeneration. Although the primary cause of disease, as well as specific signs and symptoms, vary among different forms of PMDs, there are common secondary events, including chronic inflammation, fibrosis and muscle regeneration failure, that are thought to actively contribute to disease progression [1].

The most frequent and severe form of PMD is Duchenne Muscular Dystrophy (DMD), a recessive lethal X-linked disease, caused by absence of dystrophin, a critical component of dystrophin-associated glycoprotein complex (DGC), which stabilizes the link of muscle fiber cytoskeleton to extracellular matrix. Affected boys usually lose ambulation by age 12 and die from cardiac or respiratory failure [2]. Mutations in other DGC constituents result in numerous types of muscular dystrophies. Among them, Laminin- $\alpha$ 2 deficiencies result in a severe form of muscular dystrophy—Congenital Muscular Dystrophy type 1A (CMD1A)—the second most prevalent form of congenital muscular dystrophy. Affected patients present extremely compromised neuromuscular function and muscle weakness, inability to achieve independent ambulatory capacity and premature death, also as a result of respiratory complications [3–5].

Despite innumerable advances, there is still no effective therapy for PMDs [6]. Cellular therapy strategies—involving transplantation of either stem cells or progenitors cells, in particular, mesenchymal stromal cells (MSCs)—have been

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M. Secco · C. Bueno Jr · N. M. Vieira · C. Almeida · M. Pelatti · E. Zucconi · M. Vainzof · O. K. Okamoto · M. Zatz (✉)  
Human Genome Research Center, Department of Genetic and Evolutionary Biology, Institute of Biosciences, São Paulo, Brazil  
e-mail: mayazatz@usp.br

P. Bartolini  
Department of Biotechnology,  
National Nuclear Energy Commission-IPEN-CNEN,  
São Paulo, Brazil

E. H. Miyabara  
Department of Anatomy, Institute of Biomedical Sciences,  
University of São Paulo,  
São Paulo, Brazil

the focus of intense investigation in different animal models of PMDs [7–14]. However, some of these recent studies have reported discrepant results about MSCs differentiation after delivery into skeletal muscle. Another issue that limits clinical application of MSCs is the early cell death following stem cell injection which could be partly explained by the chronic inflammation observed in dystrophic muscle. Furthermore, fibrotic tissue forms a barrier to muscle cell repopulation. Protocol optimization for MSC-based therapy has been recently reported to overcome these difficulties [12, 15–18].

Insulin-like growth factor-1 (IGF-1) is known to improve muscle regeneration by enhancing proliferation, differentiation, and survival of progenitor muscle cells [19]. IGF-1 administration or muscle-specific overexpression reduces inflammation and fibrosis in dystrophic mice, thereby contributing to tissue regeneration and reduction of muscle pathology [5, 20–24]. In addition, IGF-1 can enhance recruitment of muscle progenitor cells to sites of injury during muscle regeneration and, therefore, improve cell transplantation success [5, 25–27]. However, the combined effects of IGF-1 and MSCs therapy in animal models of muscular dystrophy have not been described yet.

Here we have investigated, for the first time, whether IGF-1 is able to promote the myogenic differentiation of MSC from umbilical cord (UC) in vitro, and if the association of both IGF-1 and human MSCs from UC could improve functional performance when administrated in a murine model for congenital muscular dystrophy type 1A—LAMA2<sup>dy2j</sup>.

## Materials and Methods

### Isolation, Culture and Characterization of MSCs from UC

All human tissue samples were donated after written informed consent of the donors. Human umbilical cord tissue was processed using methods previously described [28]. Briefly, sections of 8–10 cm of umbilical cords were internally washed with phosphate-buffered saline (PBS), supplemented with 3 % penicillin/streptomycin (Invitrogen-Gibco, Grand Island, NY, <http://www.invitrogen.com>) and immediately immersed in Dulbecco's modified Eagle's medium-low glucose (DMEM-LG; Invitrogen-Gibco) supplemented with 10 % fetal bovine serum (FBS; Invitrogen-Gibco) and 3 % penicillin/streptomycin (Invitrogen-Gibco). UCs were filled with 0.1 % collagenase (Sigma-Aldrich, St. Louis, <http://www.sigmaaldrich.com/sigma-aldrich/home.html>) in PBS and incubated at 37 °C for 15–20 min. Each UC was washed with proliferation medium (DMEM-LG supplemented with 10 % FBS and 1 % penicillin/streptomycin), and the detached cells were harvested after gentle massage of the UC. Cells were centrifuged at 300 g for 10 min, re-suspended in proliferation medium (PM), and seeded in 25-cm<sup>2</sup> flasks at a

density of  $5 \times 10^7$  cells per ml. After 24 h of incubation, non-adherent cells were removed, and PM was replaced every 3 days. Cultures were passaged repeatedly until passage 4, when we started the experiments. The remaining cells were cryopreserved in cryopreservation media (10 % dimethylsulfoxide, 90 % FBS), frozen at –80 °C in isopropanol-jacked closed container and stored in liquid nitrogen the next day.

The MSCs characterization includes immunophenotyping by flow cytometric analysis, using a panel of surface markers. For this propose, adherent cells were incubated with the following anti-human primary antibodies: CD29-PECy5, CD34-PerCP, CD31-phycoerythrin (PE), CD45-fluorescein isothiocyanate (FITC), CD90-R-PE, CD73-PE, CD13-PE, CD44-PE, CD117-PE, human leukocyte antigen (HLA)-ABC-FITC, HLA-DR-R-PE (Becton, Dickinson and Company, Franklin Lakes, NJ, <http://www.bd.com>). A total of 10,000 labeled cells were analyzed using a Guava EasyCyte flow cytometer running Guava Express-Plus software (Guava Technologies Hayward, CA, <http://www.guavatechnologies.com>). To evaluate MSCs properties, adherent cells (third passage, at 80–90 % confluence) were subjected to adipogenic, chondrogenic, and osteogenic differentiation in vitro, according to established protocols [28].

### In Vitro Myogenic Differentiation Assay

MSCs were incubated in myogenic differentiation medium (DM)—DMEM-High Glucose (DMEM-HG), supplemented with 3 % Horse Serum (HS) and 1 % penicillin/streptomycin—for 15 days, as previously described [28, 29].

The effects of IGF-1 on myogenic differentiation of MSCs were evaluated by seeding  $2 \times 10^5$  cells in 6-wells plates (Costar, Corning), with differentiation medium enriched or not with different concentrations of IGF-1 (Long R3-IGF-1; Sigma-Aldrich). At each time interval, the expression of selected myogenic markers was analyzed by Real Time RT-PCR, Western Blot and Immunofluorescence, as described in supplemental data.

### Co-Culture Experiments

DMD muscle cells from primary cultures were obtained from muscle biopsies taken for diagnostic purposes in the Human Genome Research Center, following informed consent. The biopsies were processed according to protocol described in Vieira et al. [29].

Myoblasts were cultured in proliferation medium (PM) for muscle cells—DMEM-HG supplemented with 20 % FBS and 1 % penicillin/streptomycin. Cells were maintained at 37 °C and 5 % CO<sub>2</sub>. To differentiate human myoblasts into myotubes, cells were rinsed twice with PBS and cultured in DM for 1 week. The identification of MSCs from UC in the

co-cultures with myoblasts was done by transfection with lentiviral vectors expressing GFP, produced as previously described [29].

Two types of co-culture were tested. Initially, equal amounts of myoblasts stained with DAPI (4'-6-Diamidino-2-phenylindole; Sigma-Aldrich) were mixed with GFP-positive MSCs from UC plated at 90 % confluence on culture dishes in differentiation medium supplemented or not with 50 ng/mL IGF-1. Afterwards, DMD myotubes stained with DAPI were co-cultured with GFP-positive MSCs from UC at a ratio of 3:1 of plated myotubes and MSCs, and the co-cultures were maintained in PM supplemented or not with 50 ng/mL IGF-1. Culture medium was replaced every 2–3 days. After 45 days, interaction between MSCs and DMD muscle cells was analyzed based on the presence of DAPI/GFP<sup>+</sup> myotubes by fluorescence microscopy. Dystrophin expression was analyzed by Western Blot, as described below. Co-culture control experiments were: GFP-positive MSCs from UC maintained undifferentiated; GFP-positive MSCs from UC maintained in DM for 45 days; normal control myoblasts co-cultured with GFP-positive MSCs from UC; DMD myoblasts. Normal control myoblasts were exposed to DM supplemented or not with 50 ng/mL IGF-1, as the co-culture experiments described above.

### In Vivo Experimental Design

A cohort of 46 one-month old B6.WK-*Lama2*<sup>dy/2J</sup> dystrophic mice (Jackson Laboratory) was randomly assigned into four experimental groups: untreated group (U;  $n=11$ ), IGF-1-treated group (IGF-1;  $n=9$ ), MSCs group (MSC;  $n=13$ ), and group treated with both IGF-1 and MSC (IGF-1 + MSC;  $n=13$ ). Age-matched B6.WK non-dystrophic mice were used as wild-type controls (CNT;  $n=21$ ). The analog recombinant human IGF-1 (Long R3-IGF-1; Sigma-Aldrich) was administered subcutaneously via an implanted osmotic mini-pump (model 1002; Alzet, Cupertino, CA) at a daily dose of 2 mg/kg body mass, for a period of 8 weeks. The untreated and MSCs groups received vehicle solution (100 mM acid acetic) via the same osmotic pump as a control for the administration method. During 2 months, weekly,  $1 \times 10^6$  MSCs were systemically injected in the tail vein of animals from the MSCs and IGF-1 + MSCs groups.

### Implantable Osmotic Pumps

Osmotic pumps were implanted subcutaneously. Briefly, mice were anesthetized with pentobarbitone sodium (Nembutal, 40 mg/kg i.p.; Rhone Merieux, Pinkenba, QLD, Australia) such that they were unresponsive to tactile stimuli. Surgical site was shaved and a small right paravertebral incision was made on the dorsal surface between the hind limbs.

Connective tissue below the skin was blunt dissected to create a pouch for placement of the pump. Loaded osmotic pumps (IGF-1 or vehicle solution) were inserted into this cleft with the pumping end caudally orientated. The small skin incision was closed with non-absorbable sutures and swabbed with povidone iodine solution. Since each mini-osmotic pump delivers continuously for 14 days, the 8-week administration period required each animal to have a new pump (with identical IGF-I or saline solution) implanted every 2 weeks of continuous treatment. All in vivo studies were conducted in accordance with the ethical principles in animal research adopted by the Brazilian College of Animal Experimentation ([www.cobea.org.br](http://www.cobea.org.br)) and were approved by the University of São Paulo, Institute of Biosciences Ethical Committee.

### Functional Analysis

At the end of the 8-weeks treatment, animals were subjected to muscle functional tests. In order to evaluate the grip force, animals were allowed to grab onto the Grip Strength System (model: DFE-002, San Diego Instruments, San Diego, Cal, USA) with both hind paws which measures the maximal force before the animal releases the paws of the bar. For measurement of contractile properties, mice were anesthetized with pentobarbitone sodium (Nembutal, 40 mg/kg; Rhone Merieux, Pinkenba, QLD, Australia), and *Tibialis Anterior* (TA) muscles were stimulated by supramaximal (10 V) 0.2-ms square wave pulses of 500 ms in duration at 2 Hz, delivered via two wire electrodes adjacent to the sciatic nerve. For determination of tetanic force, the frequency of electrical stimulus was elevated to 250 Hz. Muscle twitch force and tetanic force were recorded using the Biopac Systems. The muscle strength was analyzed using the AcqKnowledge 3.9.1.6. Twitch and Tetanic Forces were normalized to body mass.

After completion of all surgical procedures, mice were killed by cervical dislocation. The muscles were then excised, weight and prepared for molecular, biochemical, and histological analysis, as described in supplemental data. Blood samples were also harvested for glucose, triglycerides and cholesterol analysis, as recommended by the manufacturer (Labtest, Lagoa Santa, MG, Brazil).

### Human DNA Analysis

The presence of human DNA in the host samples was evaluated as described previously [8]. Human chromosome 7  $\alpha$ -satellite sequence (H7) was amplified by PCR (35 cycles, annealing at 59 °C). PCR products were separated by electrophoresis on 6 % acrylamide gels and stained with ethidium bromide. Non-saturated digital images were obtained using an Image Quant Imaging System (GE HealthCare). Primers sequences are listed in Table 1.

**Table 1** Primer sequences for human DNA analysis and real-time quantitative RT-PCR

| Gene             | Forward primer sequence (5'-3') | Reverse primer sequence (5'-3') |
|------------------|---------------------------------|---------------------------------|
| H7               | AGCGATTTGAGGACAATTGC            | CCACCTGAAAATGCCACAGC            |
| Endogenous       | AAATGCGGCACATCAGTTTT            | TAAGCTGGAGAGACGGTGCT            |
| Human dystrophin | GAATAAGTCAGTGACCCAGAAGAC        | TGGTGACAGCCTGTGAAATC            |
| Colla1           | GATGGTCACCCTGGAAAACC            | CACGAGCACCCCTGTGGTCC            |
| Col3a1           | AACCTGGAAGGGATGGAAAC            | GCACCAGGAGAACCATTTC             |
| TGF- $\beta$     | CCCCACTGATACGCCTGAGT            | AGCCCTGTATTCCGCTCCTT            |
| CCL2             | GGCTCAGCCAGATGCAGTTA            | GCTGCTGGTGATCCTCTTGT            |
| CCL8             | CTGGGCCAGATAAGGCTCC             | CATGGGGCACTGGATATTGTT           |
| CCL5             | ATATGGCTCGGACACCACTC            | GCACCTGCTGCTGGTGTAGA            |
| CCL3             | CGCCATATGGAGCTGACAC             | GATGAATTGGCGTGGAATCT            |
| Myf5             | CTGTCTGGTCCCGAAAGAAC            | GACGTGATCCGATCCACAATG           |
| MyoD             | TACAGTGGCGACTCAGATGC            | TAGTAGGCGGTGTCGTAGCC            |
| Myogenin         | CAGTACATTGAGCGCCTACAG           | GGACCGAACTCCAGTGCAT             |
| GAPDH            | AGGTCGGTGTGAACGGATTTG           | TGTAGACCATGTAGTTGAGGTCA         |

### Muscle Histopathology

Muscles prepared for histological analysis were immediately frozen in melting isopentane and stored in liquid nitrogen. Frozen muscles were cut into 10  $\mu$ m cross sections from the proximal to distal region using a cryostat (Criostat Micon HM505E, Walldorf, Germany). Sections of muscle were then stained with Hematoxylin and Eosin (H&E) as previously described (Dubowitz, 1985). Muscle cross sections were evaluated at 200x magnification and further analyzed on a digitizing unit connected to a computer (Image Plus, Media Cybernetic, Silver Spring, MD, USA). Cross-sectional areas (CSA), percentage of fibers with centronuclei and interstitium extent in the muscle were analyzed in blind test.

### Real-Time Quantitative RT-PCR

Total RNA was extracted from cultured cells (in vitro studies) or mouse muscle samples (in vivo studies) using TRIzol reagent (Invitrogen) as recommended by the manufacturer. First strand cDNA was produced from 1  $\mu$ g of RNA using Superscript III reverse transcription kit (Invitrogen). Real-time Quantitative RT-PCR was performed with 50 ng of cDNA and SYBR Green PCR master mix (Applied Biosystems) in 7500 Real-Time PCR System (Applied Biosystems). PCR conditions were: 94  $^{\circ}$ C for 15 s, 58  $^{\circ}$ C for 30 s, and 72  $^{\circ}$ C for 30 s for 40 cycles. The authenticity of the PCR products was verified by melt-curve analysis. Primers sequences are listed in Table 1. Samples were analyzed in triplicates, and the threshold suggested by the instrument software was used to calculate Ct. To normalize the readings we used Ct values from the GAPDH. Data are reported as a ratio of absolute mRNA

copy number of each specific gene to the absolute copy number of GAPDH.

### Western Blot Analysis

Cell (in vitro studies) or muscle (in vivo studies) proteins were extracted through treatment with 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 5 mM EDTA, 1 % Triton X-100 and 60 mM octyl glucoside. Samples were centrifuged at 13,000  $\times$  g for 10 min to remove insoluble debris. Soluble proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to nitrocellulose membranes (Hybond; Amersham). Equal loading of samples (50  $\mu$ g) and transfer efficiency were monitored using 0.5 % Ponceau S staining of the blotted membrane. Blots were blocked for 1 h in Tris-buffered saline Tween (TBST) containing 5 % powdered skim milk and reacted overnight with the following primary antibodies: rabbit monoclonal anti-Pax 7 (0,1  $\mu$ g/mL; Lifespan Bioscience), mouse monoclonal anti-MyoD (2  $\mu$ g/mL; Becton Dickinson), mouse monoclonal anti-myogenin (1:200; Abcam), rabbit anti-desmin (1:100; Sigma-Aldrich); mouse monoclonal anti-dystrophin MANDYS 104 (1:500; kindly provided by Dr. Glenn E. Morris at Center for Inherited Neuromuscular Diseases, Oswestry, Shropshire, UK), mouse monoclonal anti-TGF- $\beta$  (1,5  $\mu$ g/mL; Sigma-Aldrich), goat polyclonal anti-TNF- $\alpha$  (1:200; Santa Cruz Biotechnology), goat polyclonal anti-MURF1 (1:1,000; Santa Cruz Biotechnology), rabbit polyclonal anti-calpain1 (1:1,000; Cell Signaling), rabbit polyclonal anti-calpain 2 (1:1,000; Cell Signaling), rabbit polyclonal anti-calpastatin (1:1,000; Abcam); mouse monoclonal anti-NCX (1:1000; ABR Incorporation) and mouse monoclonal anti-SERCA1 (1:1,000, ABR Incorporation). Blots were incubated 1 h with secondary antibodies

(horseradish peroxidase-conjugated antibody) and immunoreactive bands were detected with ECL chemiluminescence detection system (GE Healthcare). Quantitative analysis of human dystrophin at the host muscle was performed with ImageJ software (<http://rsb.info.nih.gov/ij/>), considering the Myosin at the Ponceau staining as a load protein control.

#### Immunofluorescence

Cells grown in 1-chamber slides (Nalgene-NUNC) were fixed in 4 % paraformaldehyde in PBS for 20 min at 4 °C and permeabilized with 0,05 % Triton X-100 in PBS for 5 min. Non-specific binding was blocked with 10 % FBS in PBS for 1 h at room temperature. Cells were incubated with primary antibody overnight at 4 °C and with secondary antibody for 1 h at room temperature. The following primary antibodies were used: Anti-Desmin (1:20, Sigma-Aldrich), Anti-Skeletal Myosin (1:20, Sigma), combined with goat anti-rabbit IgG secondary antibody, Cy3-Conjugated (1:100, Chemicon). The fluorescence signal was examined in Axiovert 200 (Carl Zeiss) and in ApoTome Imaging System (Carl Zeiss).

#### Statistical Analysis

All values are presented as means  $\pm$  standard error (SE). Student's *t* test was used to comparison between two groups. Statistical significance was considered as  $p < 0.05$ .

## Results

### IGF-1 Enhances Myogenic Differentiation of MSCs

The evaluation of MSCs properties included immunophenotyping by flow cytometry analysis, using a panel of surface markers. At passage four, adherent cells from UC were negative for CD31 (endothelial cell marker), CD34, CD45, CD117 (hematopoietic cell markers), and HLA-DR (human leukocyte differentiation antigen class II) expression, but positive for the expression of CD29, CD44, CD90, (adhesion markers) CD73, CD13 (mesenchymal markers), and HLA-ABC (human leukocyte differentiation antigen class I; data not shown). Cell plasticity was assessed by in vitro differentiation capacity, after 3 weeks of induction using appropriate conditions. Adipogenic, chondrogenic and osteogenic differentiation were demonstrated by the presence of lipid vacuoles, mucopolysaccharide-rich extracellular matrix and calcium deposits, respectively (data not shown). These results confirmed the mesenchymal nature of the isolated cells as well as their multipotent potential.

In order to verify if IGF-1 was capable of promoting a more efficient myogenesis of MSCs, human MSCs from UC were incubated in myogenic differentiation medium (DM) supplemented with different concentrations of IGF-1. Prior to myogenic induction (Control), MSCs were negative or expressed low levels of muscle lineage markers, as measured by immunofluorescence (IF) and western blot (WB) analysis (Fig. 1a-h). Continuous exposure of MSCs to DM (HS) supplemented or not with IGF-1 over 15 days resulted in the development of Desmin and MyHC-positive fibers (Fig. 1g). Myogenic differentiation was more prominent in MSCs cultured with DM containing IGF-1 than those cells cultured in DM only, as revealed by up-regulation of earlier myogenic markers, such as Pax7, MyoD and Myogenin, as well as markers of terminal muscle differentiation, including, MyHC, Desmin and Dystrophin (Fig. 1a-f).

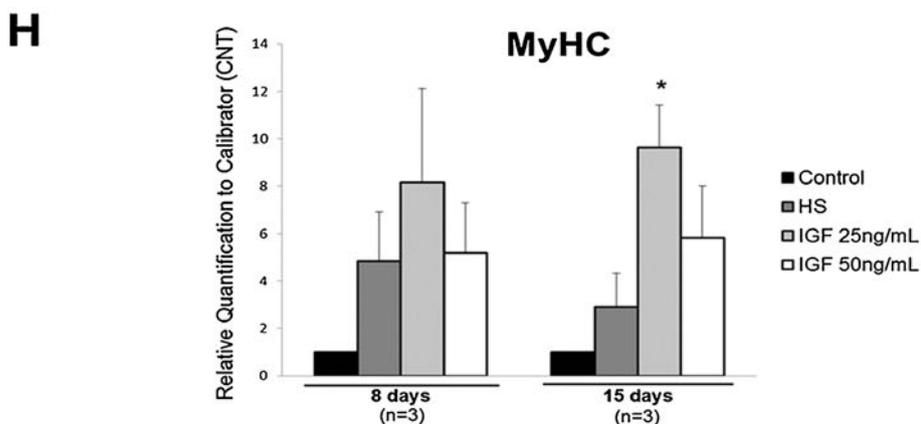
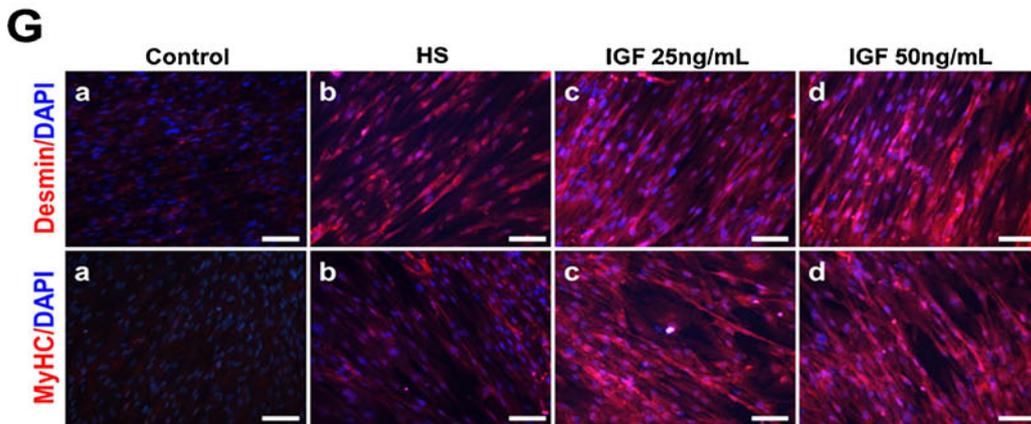
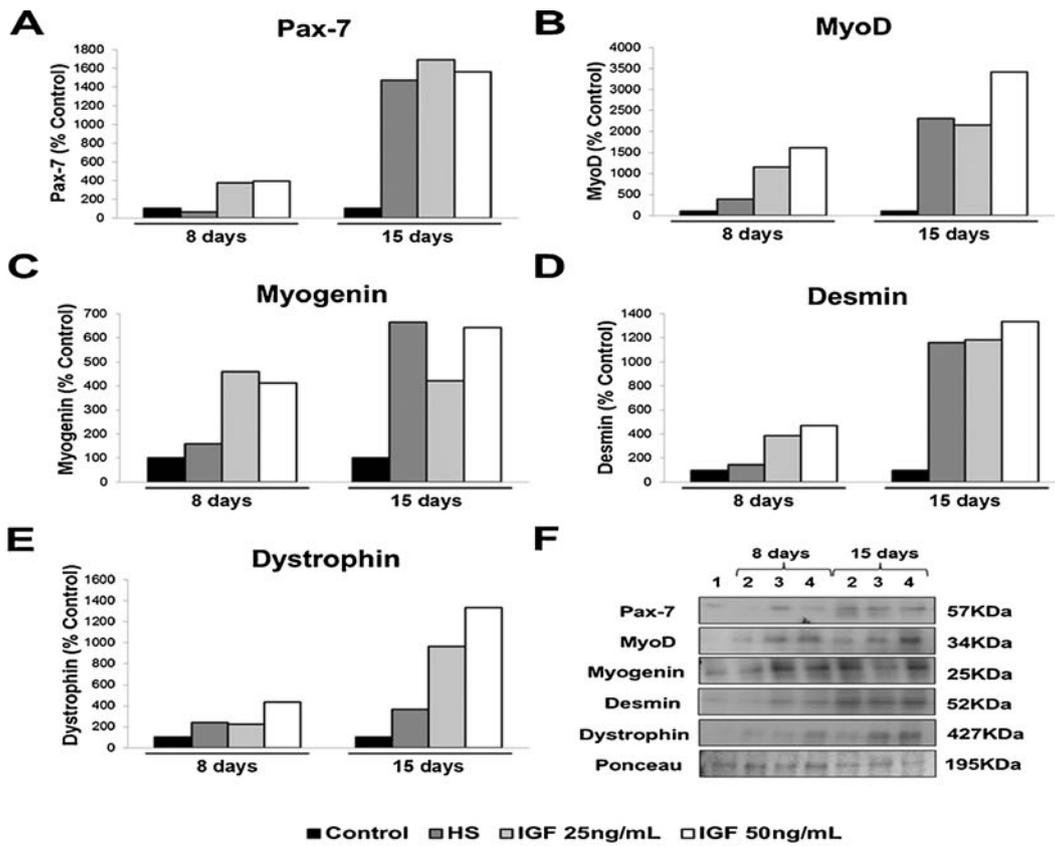
MSCs exposed to 50 ng/mL IGF-1 displayed a gradual increase in the expression of Pax-7 (~4 and 16-fold, respectively), MyoD (16- and 34-fold) and Myogenin (4 and 6-fold), after 8 and 15 days, respectively. Similarly, expression of later myogenic markers also increased over time. When compared with non-induced cells, MSCs exposed to IGF-1 showed about 5 and 13-fold increase in the expression of Desmin, as well as Dystrophin, at 8 and 15 days, respectively (Fig. 1a-f). These results were confirmed by Real-time Quantitative RT-PCR that showed a significant increase in MyHC expression only in MSCs treated with IGF-1 for 15 days, in comparison with MSCs treated with DM alone (Fig. 1h).

### IGF-1 Enhances Interaction Between MSCs and DMD Muscle Cells and Restores Dystrophin Expression

MSCs expressing GFP were co-cultured with primary GFP-negative DMD myoblasts or myotubes previously stained with DAPI (Fig. 2a). MSCs from UC were stably transfected with GFP in order to distinguish them from DMD muscle cells. About 80–90 % of cells were GFP-positive and GFP expression did not decline during culture passages (Fig. 2b).

After 45 days of co-culture, multinucleated myotubes were observed with areas of GFP-positive syncytia in the resulting cultures (data not shown). In both co-cultures, GFP-positive syncytia presenting at least one DAPI stained nucleus (Fig. 2b).

Finally, we analyzed dystrophin protein levels in the two different co-cultivation assays. As expected, there was no dystrophin expression in DMD muscle cells but its expression was restored in DMD muscle cells co-cultured with MSCs (Fig. 2c). More interestingly, IGF-1 treatment induced a robust increase in the levels of dystrophin protein in both DMD/normal myoblast and myotubes co-cultures (Fig. 2c), suggesting that MSCs from UC participate in the



**Fig. 1** IGF-1 enhances myogenic differentiation of MSCs. **a-e** Western Blot analysis of Pax-7, MyoD, Myogenin, Desmin, and Dystrophin expression in non-differentiated MSCs (control) and MSCs cultured in differentiation medium (HS; horse serum) supplemented or not with different concentrations of IGF-1 (25 ng/mL or 50 ng/mL), for 8 and 15 days. Data correspond to optical density of respective protein bands, assessed by ImageJ software. Results are expressed as percentage from control; **f** Representative Western Blot bands. *Samples:* 1) Non-differentiated MSCs (control); 2) MSCs cultured in differentiation medium alone (HS); 3) MSCs cultured in differentiation medium supplemented with 25 ng/mL IGF-1; 4) MSCs cultured in differentiation medium supplemented with 25 ng/mL IGF-1; **g** Representative myogenic differentiation images assessed by immunofluorescence. MSCs treated with either 25 or 50 ng/mL IGF-1 (*c,d*) were more strongly labeled with anti-human Desmin and MyHC antibodies (*red*) than cells cultured in the absence of IGF-1 (*a,b*). Counterstaining with DAPI (*blue*) was used to identify all nuclei. Scale bar=100  $\mu$ m; **h** Real-time Quantitative RT-PCR analysis of MyHC (Myosin Heavy Chain) in non-differentiated MSCs (control) or MSCs cultured in differentiation medium (HS) supplemented or not with different concentrations of IGF-1 (25 ng/mL or 50 ng/mL), for 8 and 15 days. The number of cell lines analyzed in each group is shown in *parentheses* and data are presented as mean  $\pm$  SE. \* $P$ <0.05 versus MSCs cultured in HS; Student's *t* test

generation of human myotubes through cellular fusion and that IGF-1 actively contributes to this process.

#### IGF-1 and MSCs Treatment Do Not Alter Basic Parameters of LAMA2<sup>dy2j</sup> Injected Mice

As presented in Table 2, continuous administration of IGF-1 for 8 weeks or MSCs injections or both treatments combined did not alter morphometric properties such as body, heart, liver, *Tibialis Anterior* (TA) mass, as well as the triglycerides and glucose values, compared with untreated dystrophic LAMA2<sup>dy2j</sup> mice. Only cholesterol levels were significantly lower in the group that received both IGF-1 and MSCs, when compared with the untreated dystrophic group (Table 2).

#### Combination of IGF-1 and MSCs Improves Skeletal Muscle Strength in LAMA2<sup>dy2j</sup> Mice

The effects of IGF-1 and MSCs on disease progression were investigated by comparing the grip force of LAMA2<sup>dy2j</sup> mice. Untreated LAMA2<sup>dy2j</sup> animals presented a significant decrease of 43 $\pm$ 12 % in their grip force, 2 months after the experiment onset. Mice treated with either IGF-1 or MSC alone also worsened significantly their performance in about 23 $\pm$ 8 % and 25 $\pm$ 12 %, respectively. Only the group of mice treated with both IGF-1 and MSCs presented a significant improvement of 12 $\pm$ 9 % in grip force when evaluated 2 months post-treatment, compared with either untreated, IGF-1 or MSC-treated mice (Fig. 3a). IGF-1 + MSC group also presented a significant increase in both muscle twitch and tetanic force, similar to those observed in control muscle

(Fig. 3b, c). Altogether, these results indicate that only the combination of both IGF-1 and MSCs restored skeletal muscle strength in dystrophic mice.

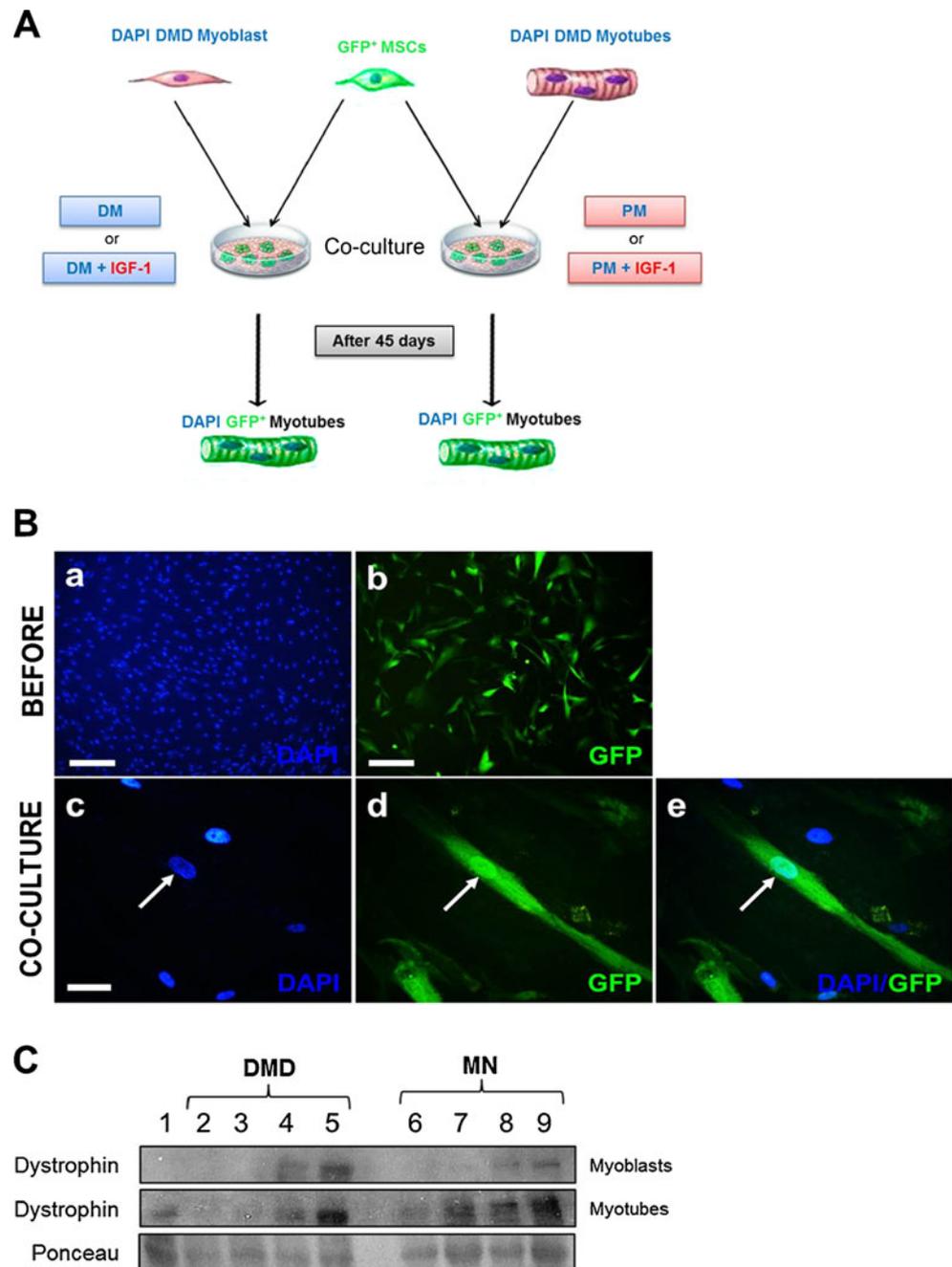
#### MSCs Injected Systemically in LAMA2<sup>dy2j</sup> Mice Were Able to Reach the Skeletal Muscle but Did Not Differentiate into Muscle Cells, Even When Associated with IGF-1

Human DNA was found in TA muscle of MSCs-injected mice, with or without IGF-1 co-treatment, which indicates that MSCs from UC were not rejected and were able to migrate to dystrophic muscle after systemic delivery (Fig. 4a). Since we had previously found that IGF-1 increased myogenic differentiation of MSCs in vitro, we next verified whether the improvement in muscular strength observed in the IGF-1 + MSC-treated mice could be due to this effect. Antibody against human-laminin- $\alpha$ 2 recognizes the LAMA2<sup>dy2j</sup> mutant laminin. Therefore, we evaluated the presence of dystrophin, using a specific human-dystrophin antibody. No human dystrophin was found in muscles of treated animals through WB analysis (Fig. 4b). We also confirmed these results with RT-PCR using specific primers to human dystrophin, which showed no transcript expression (data not shown). These results suggest that MSCs from UC—differently from results obtained in vitro—were not able to differentiate into muscle cells in vivo.

#### Combination of MSCs and IGF-1 Reduces Fibrosis in LAMA2<sup>dy2j</sup> Dystrophic Muscle

To better understand the effects of both IGF-1 and MSCs in dystrophic muscle, we stained muscle sections with hematoxylin and eosin (H&E) and analyzed the morphological characteristics of LAMA2<sup>dy2j</sup> pathology (Fig. 5a). H&E staining of untreated dystrophic TA muscle showed typical dystrophic features, including abnormal variation of fiber size, central nucleation and excessive interstitial fibrosis, when compared with control muscle (Fig. 5a). Administration of IGF-1, MSC or IGF-1 + MSC did not alter significantly the fiber CSA neither the percentage of central nucleation in the TA muscles, when compared with the untreated group (Fig. 5b, c). Although the IGF-1 group presented a moderate reduction in the interstitium, only the group of mice treated with IGF-1 + MSC showed a significant decrease in the interstitium extent. Muscles of MSC-treated mice, on the other hand, showed an average amount of interstitium similar that observed in LAMA2<sup>dy2j</sup> untreated muscles (Fig. 5d). To verify if the amount of interstitium was related with the extent of fibrosis in LAMA2<sup>dy2j</sup> mice, we performed a picro-sirius red staining. Only mice treated with IGF-1 + MSC had a significant reduction in the extent of fibrosis, when compared with the untreated dystrophic group (Fig. 5e).

**Fig. 2** IGF-1 enhances interaction between MSCs and DMD muscle cells and restores dystrophin expression. **a** Schematic representation of co-culture experiments; **b** Images from fluorescence microscopy show GFP-positive (*green*) MSCs (*a*) and DAPI (*blue*) DMD muscle cells (*b*), before the experiments. After 45 days of co-culture, interaction between Mesenchymal Stromal Cells (MSCs) and Duchenne Muscular Dystrophy (DMD) muscle cells was confirmed by the presence of DAPI/GFP<sup>+</sup> myotubes (*c-e*). Arrows indicate nuclei of a DAPI/GFP<sup>+</sup> myotubes (*c-e*); **c** Western Blot analysis of Dystrophin expression; *First Line*: Co-culture of *myoblasts* with MSCs maintained in differentiation medium supplemented or not with IGF-1; *Second Line*: Co-culture of *myotubes* with MSCs maintained in proliferation medium supplemented or not with IGF-1; *Samples*: 1) GFP-positive MSCs; 2) DMD muscle cells; 3) DMD muscle cells culture in medium supplemented with IGF-1; 4) Co-culture of MSCs with DMD muscle cells; 5) Co-culture of MSC with DMD muscle cells maintained in medium supplemented with IGF-1; 6) Normal muscle cells; 7) Normal muscle cells culture in medium supplemented with IGF-1; 8) Co-culture of MSCs with normal muscle cells; 9) Co-culture of MSCs with normal muscle cells maintained in medium supplemented with IGF-1



We also examined the expression patterns of specific proteins related with the fibrous tissue formation, including type I (Col1a1) and III (Col3a1) fibrillar collagens and transforming growth factor beta 1 (TGF- $\beta$ 1). WB and RT-PCR analysis showed that IGF-1 or MSCs alone did not alter TGF- $\beta$  expression. However the expression of TGF- $\beta$  protein was prominently down-regulated in the group treated with IGF-1 + MSC. Consistent with the overall decreased in TGF- $\beta$  expression, the levels of Col1a1 and Col1a3 expression were also significantly decreased in mice treated with IGF-1 + MSC when compared with the untreated group. However, when analyzing the IGF-1 or MSC administration

individually, there was a statistical decreased of Col1a1 only in the IGF-1 treated group. These data suggest that the combined, but not single, use of IGF-1 and MSCs reduces fibrosis in dystrophic muscle (Fig. 5f-j).

#### Combination of MSC and IGF-1 Modulates Inflammation through Reduction of Inflammatory Molecules in Skeletal Muscle of LAMA2<sup>dy/2j</sup> Mice

To verify whether improvement in muscular strength observed in the IGF-1 + MSC group was also associated with modulation of inflammatory response, we examined expression

patterns of specific cytokines and chemokines related with infiltration of inflammatory cells into damaged muscle. TNF- $\alpha$  levels, an important mediator of the early inflammatory response, were significantly lower in the IGF-1 + MSC treated animals, when compared with untreated LAMA2<sup>dy/2j</sup> mice. Conversely, higher TNF- $\alpha$  levels were found in mice treated with either IGF-1 or MSCs individually, relative to control levels (Fig. 6a, b). A significant decrease in CCL2 expression was observed in IGF-1, MSC, and IGF1 + MSC-treated mice, when compared with untreated dystrophic mice (Fig. 6c). Notably, CCL8 expression, as well as, RNA levels of CCL3 were significantly decreased only in IGF-1 + MSC treated mice (Fig. 6d, f). IGF-1 only and IGF-1 + MSC groups had significantly reduced levels of CCL5 compared with untreated group, while MSC group presented an increased expression of this same chemokine (Fig. 6e).

#### Association of Both MSC and IGF-1 Contributes to the Regeneration Process in Lama2<sup>dy/2j</sup> Dystrophic Muscle

To analyze if the mild inflammatory response observed in IGF-1 + MSC group contributed to enhanced muscle regeneration, we examined the expression levels of proteins involved in myogenesis, including myogenin, MyoD, and MRF4 (Fig. 7a-f). Significant increase in myogenin protein levels were found in IGF-1, MSC and IGF-1 + MSC treated LAMA2<sup>dy/2j</sup> mice when compared with untreated dystrophic mice (Fig. 7a). However, significantly higher levels of MyoD protein were only found in mice subjected to IGF-1 + MSC treatment (Fig. 7b). Transcript levels of myogenin, MyoD and MRF4 were similar among groups (Fig. 7d-f). The increased myogenin and MyoD expression may contribute to muscle regeneration and, therefore, improve the muscular strength observed in IGF-1 + MSCs co-treated mice.

#### Combination of MSC and IGF-1 Affects Expression of Proteins Related with Proteolytic Systems in Muscle of LAMA2<sup>dy/2j</sup> Mice

Since protein breakdown has been postulated to be one of the intrinsic factors responsible for muscle wasting, we studied the expression of proteins related to proteolytic systems. As shown in Fig. 8a, only the combination of both IGF-1 + MSCs significantly decreased levels of Murf-1, an important protein of proteolytic pathways activated in several pathologies, in comparison with the LAMA2<sup>dy/2j</sup> untreated mice. We also examined the expression of calpain-1, -2 and calpastatin, since the calpain/calpastatin system-mediated protein degradation is an important proteolytic pathway activated during myofiber degeneration. Calpain-1 and calpain-2 levels remained unchanged but, significantly higher levels of calpastatin—an endogenous calpain inhibitor—were found in mice treated with IGF-1+MSC, compared with untreated dystrophic mice (Fig. 8).

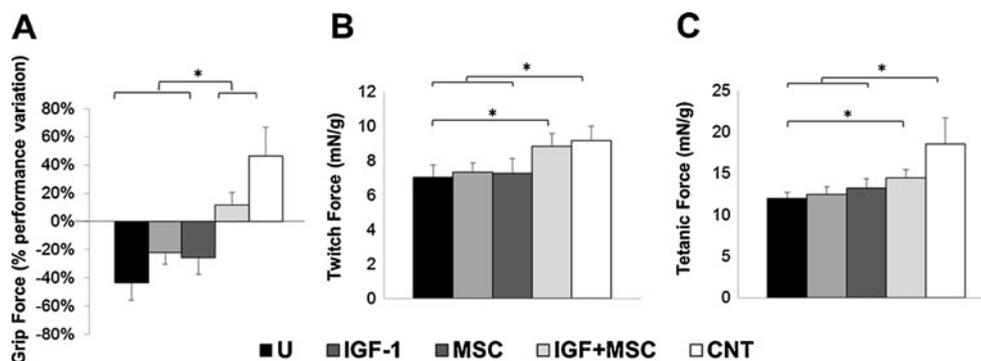
#### Discussion

IGF-1 has been shown to be responsible for activating both myoblast proliferation and subsequent myogenic differentiation, two processes that are crucial for muscle regeneration [19, 27]. Here we demonstrated, for the first time, that IGF-1 is also able to induce a rapid and efficient myogenic conversion of human MSCs from UC, with formation of mature myotubes, in vitro. This IGF-1-induced myogenic differentiation was accompanied by sequential expression of early, intermediate, and late MRFs, such as Pax7, MyoD, and Myogenin, respectively, which is comparable to what occurs during embryonic development and skeletal muscle regeneration. Interestingly, we also demonstrated that continuous IGF-1 exposure resulted in a robust increase in later myogenic markers levels (such as Desmin, MyHC, and Dystrophin) in MSCs cultures.

**Table 2** Basic parameters of dystrophic LAMA2<sup>dy/2j</sup> mice after treatment with IGF-1 and MSC

|                             | Untreated (n=7) | IGF-1 (n=8)  | MSC (n=8)    | IGF-1 + MSC (n=8) | Control (n=7) |
|-----------------------------|-----------------|--------------|--------------|-------------------|---------------|
| Body mass (g)               | 20.61±0.70      | 19.88±0.30   | 18.98±0.39*  | 20.73±0.84        | 25.34±0.78**  |
| TA mass/body mass (mg/g)    | 1.47±0.05       | 1.40±0.04    | 1.47±0.05    | 1.46±0.04         | 1.90±0.03**   |
| Heart mass/body mass (mg/g) | 4.97±0.12       | 5.23±0.12    | 5.16±0.28    | 5.27±0.18         | 4.60±0.13**   |
| Liver mass/body mass (mg/g) | 52.55±1.87      | 55.96±2.58   | 55.35±2.03   | 53.41±2.64        | 45.79±1.49**  |
| Glucose (mg/dL)             | 132.04±35.68    | 171.08±27.69 | 204.97±28.88 | 180.10±36.45      | 205.23±28.19  |
| Triglycerides (mg/dL)       | 157.86±23.31    | 128.90±13.03 | 159.13±37.83 | 126.06±14.74      | 117.07±12.71  |
| Cholesterol (mg/dL)         | 101.15±7.50     | 102.57±6.48  | 90.22±7.73   | 83.53±4.98***     | 93.80±6.27    |

The number of animals in each group is shown in parentheses and data are presented as mean ± SE. *Tibialis Anterior* (TA), heart and liver mass was normalized to body mass.\* $P$ <0.05 versus untreated group; \*\* $P$ <0.05 versus untreated and treated LAMA2<sup>dy/2j</sup> dystrophic mice; \*\*\* $P$ <0.05 versus untreated and IGF-1 treated mice; Student's  $t$  test



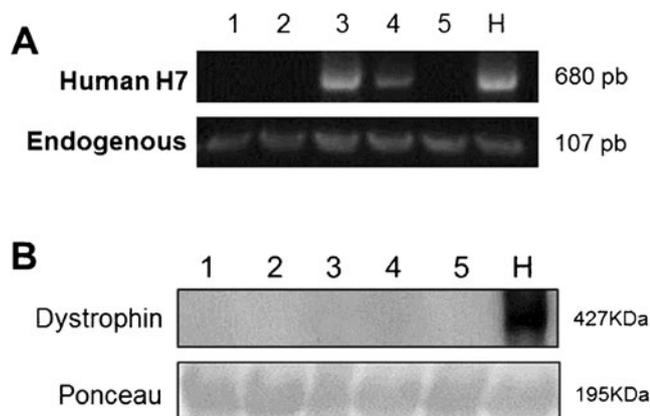
**Fig. 3** Association of IGF-1 and MSCs improves skeletal muscular strength in LAMA2<sup>dy/2j</sup> dystrophic mice. **a** Grip Force indicated as performance variation before and after the treatments **b,c** Muscle Twitch and Tetanic Force determined at 1 Hz and 250 Hz of electrical stimulus frequency, respectively, in untreated dystrophic mice (U;  $n=11$ ),

IGF-1 treated mice (IGF-1;  $n=9$ ), MSC treated mice (MSC;  $n=13$ ) and both IGF-1 and MSC treated mice (IGF-1 + MSC;  $n=13$ ). Twitch and Tetanic Forces were normalized to body mass. Data are presented as mean  $\pm$  SE. \* $P<0.05$ ; Student's  $t$  test

Moreover, we showed that IGF-1, when added to co-cultures of MSCs from UC and DMD muscle cells, enhances the fusion of MSCs with myoblasts as well as with myotubes and improves restoration of dystrophin expression. These results further reinforce two mechanisms by which IGF-1 signaling contributes to muscle regeneration: de novo generation of muscle-specific cells by the fusion of myoblasts—a process known as hyperplasia—or the fusion of myoblasts with preexisting muscle fibers and myofiber maturation, characterizing the process known as hypertrophy. Moreover, in vitro expression of dystrophin supports the hypothesis that MSCs from UC, when associated with IGF-1 have the potential to be used for Duchenne muscular dystrophy therapy and, therefore, should be further tested as a potential therapeutic approach.

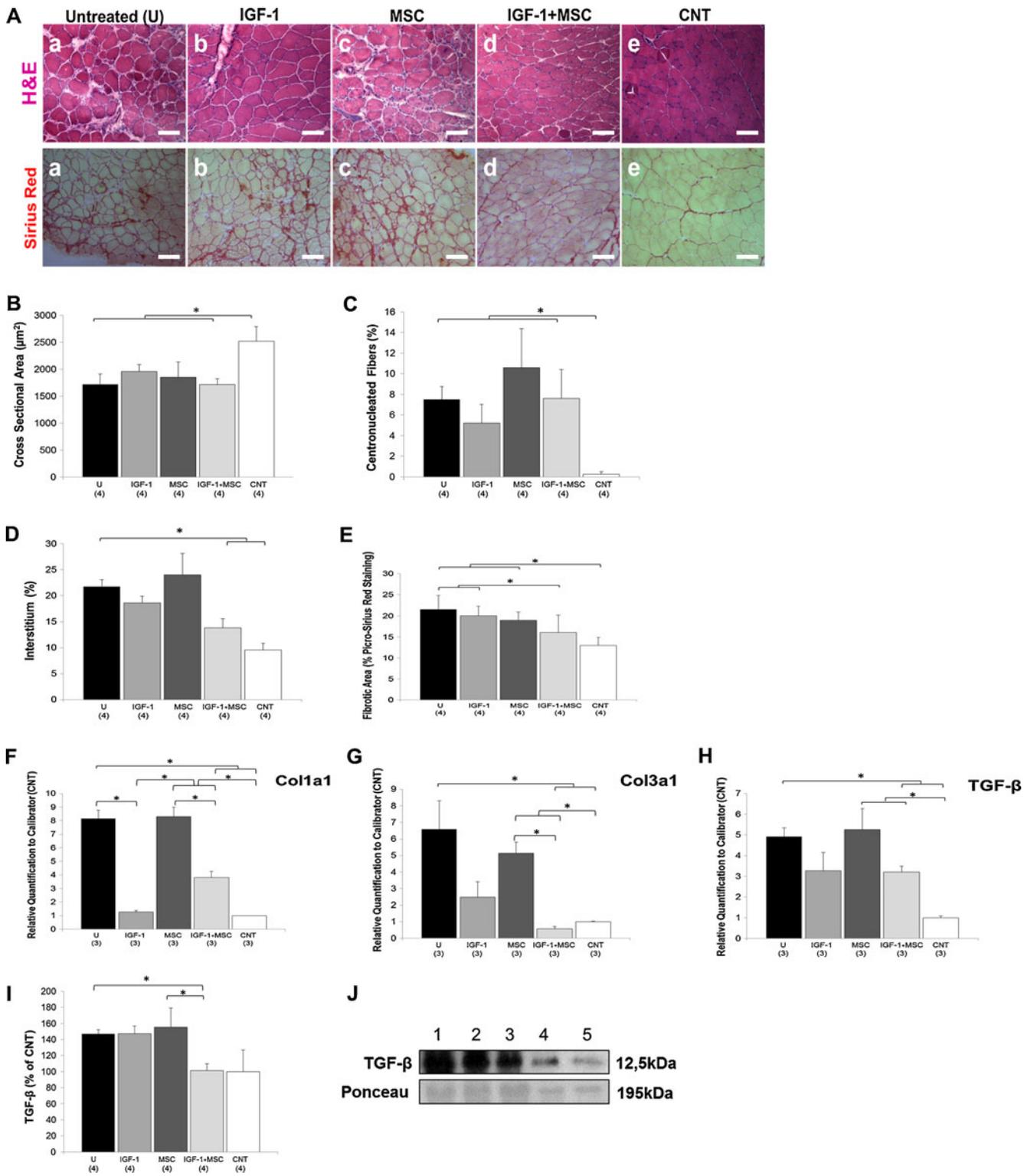
Previous studies have obtained encouraging results following IGF-1 treatment or transplantation of different stem cell preparations, independently, into mouse models of muscular dystrophy [5, 7–14, 20–24]. However, we are not aware of any previous pre-clinical study testing the IGF-1 effects on MSCs transplantations. Moreover, most of these studies were performed in DMD murine model—*mdx*—that has no evident muscular weakness and therefore is not the best model to assess potential functional effects of different therapeutic approaches. On the other hand, mouse models for congenital muscular dystrophy, such as LAMA2<sup>dy/2j</sup> mice used here, presents an extremely severe phenotype [30].

Our data demonstrated that the combined treatment of IGF-1 with MSCs injected systemically enhances muscle repair and has a clear beneficial effect in LAMA2<sup>dy/2j</sup> treated dystrophic mice. To further understand the effects of IGF-1 associated with MSCs in muscle repair, we firstly analyzed if IGF-1 was able to promote muscle engraftment and myogenic differentiation of MSCs after their systemic delivery.



**Fig. 4** Systemic injection of MSCs from UC in dystrophic mice were able to reach the skeletal muscle but did not differentiate into muscle cells, even when associated with IGF-1. **a** PCR analysis for human chromosome 7  $\alpha$ -satellite sequences (H7) or endogenous sequence (Endogenous) in the *Tibialis Anterior* (TA) muscle from untreated and treated mice. **b** Western blot analysis of human dystrophin in TA muscle from mice. *Samples*: 1) Untreated dystrophic mice; 2) IGF-1 treated mice; 3) MSC treated mice; 4) IGF-1 + MSC treated mice; 5) Non-dystrophic mice (control-CNT); H) Human DNA or protein used as a positive control

**Fig. 5** Association of both MSCs and IGF-1 reduces fibrosis in LAMA2<sup>dy/2j</sup> dystrophic muscle. **a** Representative H&E and Picrosirius Red-stained cross sections of *Tibialis Anterior* (TA) muscle from untreated or treated mice; **b-e** Diagram showing the quantitative analysis of Cross-Sectional Area, Centronucleated Fibers, Interstitium and Fibrotic Area percentage (**c,d,e**); **f-h** Real-time Quantitative RT-PCR analysis of Col1a1, Col3a1, and TGF- $\beta$  expression in muscle samples from untreated dystrophic mice (U), IGF-1 treated mice (IGF-1), MSC treated mice (MSC), both IGF-1 and MSC treated mice (IGF-1 + MSC), in comparison with non-dystrophic mice (control; CNT) expression; **i** Western Blot analysis of TGF- $\beta$  expression in muscle samples from untreated and treated mice, compared with the control (CNT). Data correspond to optical density of respective protein bands, assessed by ImageJ software; **j** Representative Western Blot bands. *Samples*: 1) Untreated dystrophic mice; 2) IGF-1 treated mice; 3) MSC treated mice; 4) IGF-1 + MSC treated mice; 5) Non-dystrophic mice (CNT). The number of animals analyzed in each group is shown in *parentheses* and data are presented as mean  $\pm$  SE. \* $P<0.05$ ; Student's  $t$  test



Human MSCs from UC can reach and engraft into recipient dystrophic muscle cells after systemic delivery, even without immunosuppression. Since we did not find human dystrophin in skeletal muscle of MSCs injected mice, it seems unlikely that MSCs have differentiated into muscle cells. However, dystrophin is a later myogenic marker, therefore we cannot exclude the possibility that the injected cells were in earlier stages of myogenesis. Most importantly, the functional recovery found in animals treated with IGF-1 + MSC reinforces the hypothesis that dystrophin expression may not be required for a significant functional amelioration. Animal models for DMD, such as the *mdx* mice and also larger exceptional animals such as the golden retriever muscular dystrophy dogs described previously, have shown that muscle can be functional despite the absence of dystrophin [31, 32]. On the other hand, Gang et al. [9] reported that MSCs restored dystrophin expression when injected into dystrophin-deficient mouse, but this effect was not accompanied by functional recovery. Conversely, our group demonstrated that human MSCs from UC injected in a *SJL* mice model were able to reach the muscle and promote a therapeutic benefit in injected animals, despite the lack of human dystrophin expression [10]. Supporting these findings, several evidences have indicated that terminal differentiation is not a major determinant for success of stem cell therapy and that MSCs contribute to tissue repair through production of trophic factors, including growth factors, cytokines and antioxidants, some of which providing the basis for their capacity to modulate inflammatory and/or immune responses [9, 13, 33–36]. In order to explore how the association of IGF-1 and MSCs could enhance muscle repair, we analyzed aspects related with muscle inflammation, fibrosis and regeneration.

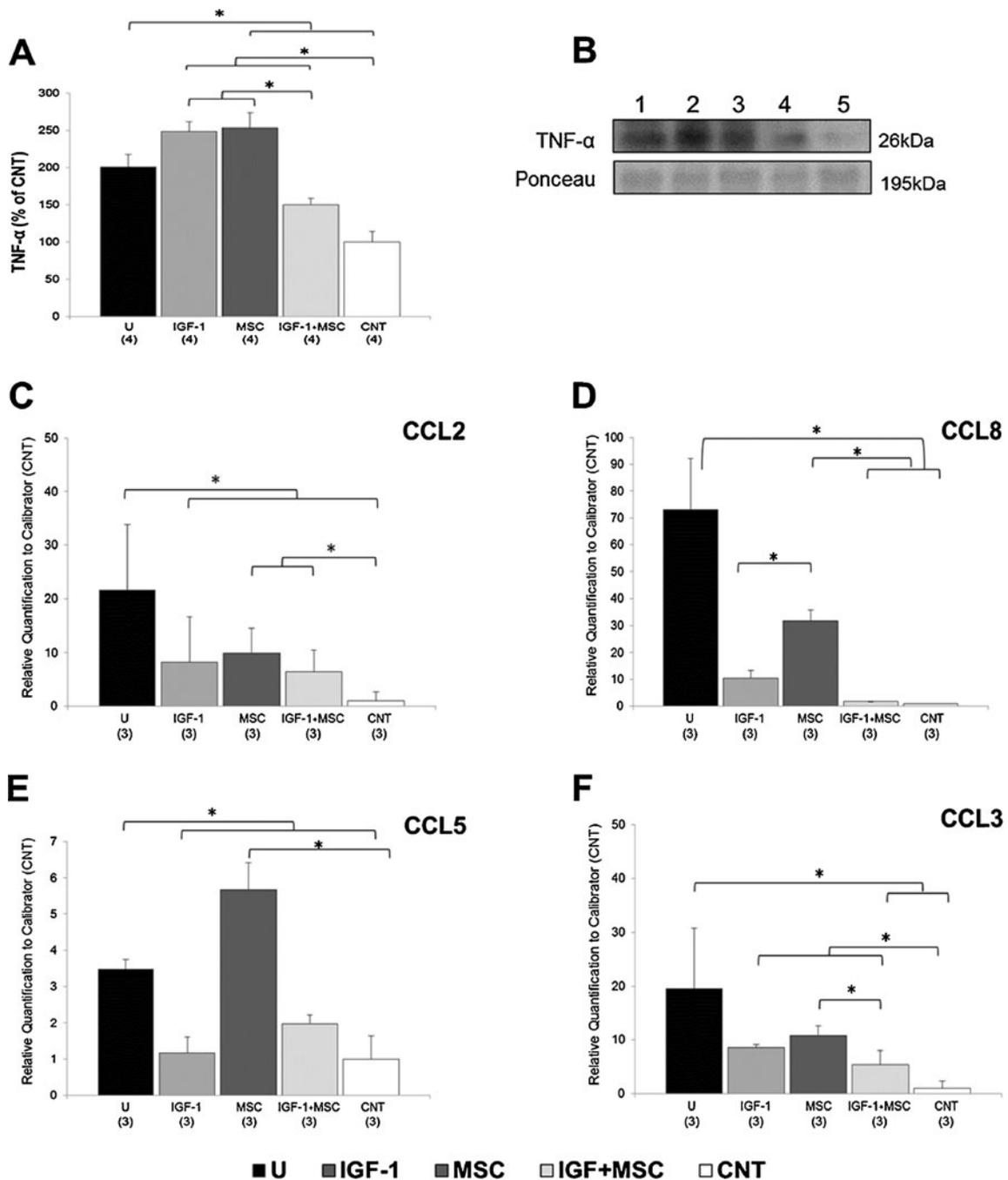
Inflammation is clearly a critical component of the regenerative process, since it contributes to removal of necrotic material and also to secretion of several cytokines and growth factors stimulating progenitor muscle cells activation [26]. Nevertheless, the inflammatory response must be resolved to allow muscle repair and prevent replacement of skeletal muscle by fibrotic tissue [26]. Our results demonstrated that only mice treated with IGF-1 + MSCs presented a significant decrease in expression of pro-inflammatory cytokines, such as TNF- $\alpha$  and the CC chemokines. Several independent investigations support the anti-inflammatory properties of IGF-1 as well as MSCs [13, 26, 33]. It is possible that additive effects due to combination of IGF-1 and MSCs are responsible for the significant reduction of TNF- $\alpha$  and the CC chemokines observed here.

Since these cytokines and chemokines are associated with sustained influx of inflammatory cells into damaged muscle, switch from acute to a chronic inflammatory process and, consequently, onset of fibrotic process [26], it is probably that their down-regulation could contribute to accelerate the timing of inflammation and to limit fibrosis. In fact, muscle of

IGF + MSCs treated animals presented evidences of fibrosis reduction, such as decreased interstitium in muscle sections and down-regulation of specific proteins involved in fibrous tissue formation. It has been previously demonstrated that IGF-1 prevents fibrosis in several pathological conditions [26], but more recent studies have reported that IGF-1 alone was not able to alter the extent of collagen infiltration in dystrophic murine models [5, 24]. Similarly, there are conflicting results about MSCs anti-fibrotic properties; in opposition to the evidences showing that MSCs are able to reduce fibrosis, some authors have demonstrated that MSCs induce expression of TGF- $\beta$  [36–38]. Interestingly, our results demonstrated that the combination of IGF-1 and MSCs is capable of minimizing this fibrotic process. Considering that no effective pharmacotherapy exists to attenuate muscle necrosis or fibrosis in patients with muscular dystrophy without considerable side effects, our results are of utmost clinical importance.

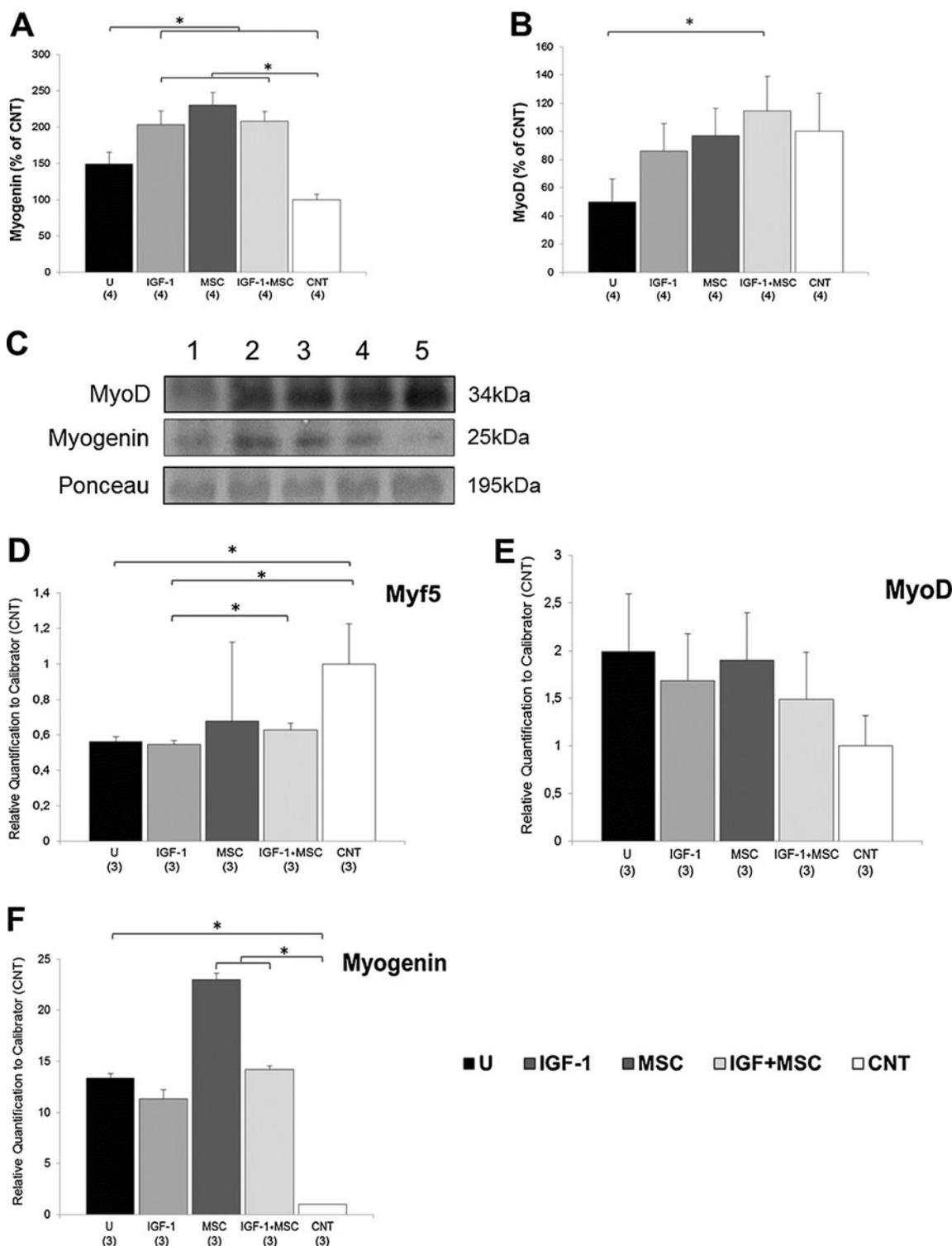
If decreased inflammatory response and fibrosis might create a qualitatively different environment for sustaining more efficiently the muscle regeneration, regulation of myogenic factors could be responsible for the muscle repair observed in animals treated with both IGF-1 and MSCs. Interestingly, we observed that muscle regenerative markers such as MRFs, MyoD and Myogenin were expressed at significantly higher levels in mice treated with IGF-1 + MSC. Besides increased muscle regeneration capacity, we also observed significant changes in expression of proteins related with proteolytic systems, including Murf-1 and calpastatin, in muscles of LAMA2<sup>dy/2j</sup> mice treated with IGF-1 + MSCs. Since rapid muscle mass loss is primarily due to accelerated protein degradation, down-regulation of proteins responsible for proteolysis in IGF-1 + MSC-treated mice could help prevent muscle wasting [39]. Consistent with our findings, previous studies have shown that IGF-1 can down-regulate Murf-1 expression, as well as, up-regulate calpastatin, which are important modulators of proteolytic systems during muscle atrophy [40]. However, this is the first report addressing the effects of MSCs alone or combined with IGF-1 in skeletal muscle wasting induced by increased protein breakdown.

In short we suggest that IGF-1 is able to promote a robust myogenic differentiation of MSCs in vitro and enhance the interaction between MSCs and DMD muscle cells in co-cultures. Furthermore, our in vivo studies showed, for the first time, that combination of IGF-1 + MSCs improves muscle function and reduces skeletal muscle pathology in a murine model of congenital muscular dystrophy. These effects involve modulation of the inflammatory response and reduction of fibrosis. Moreover, the fact that we could not find specific human muscle protein in skeletal muscles of injected mice suggests that improvement of muscle regeneration in IGF-1 + MSC group is mainly correlated with enhanced activation of progenitor and/or satellite cells rather than



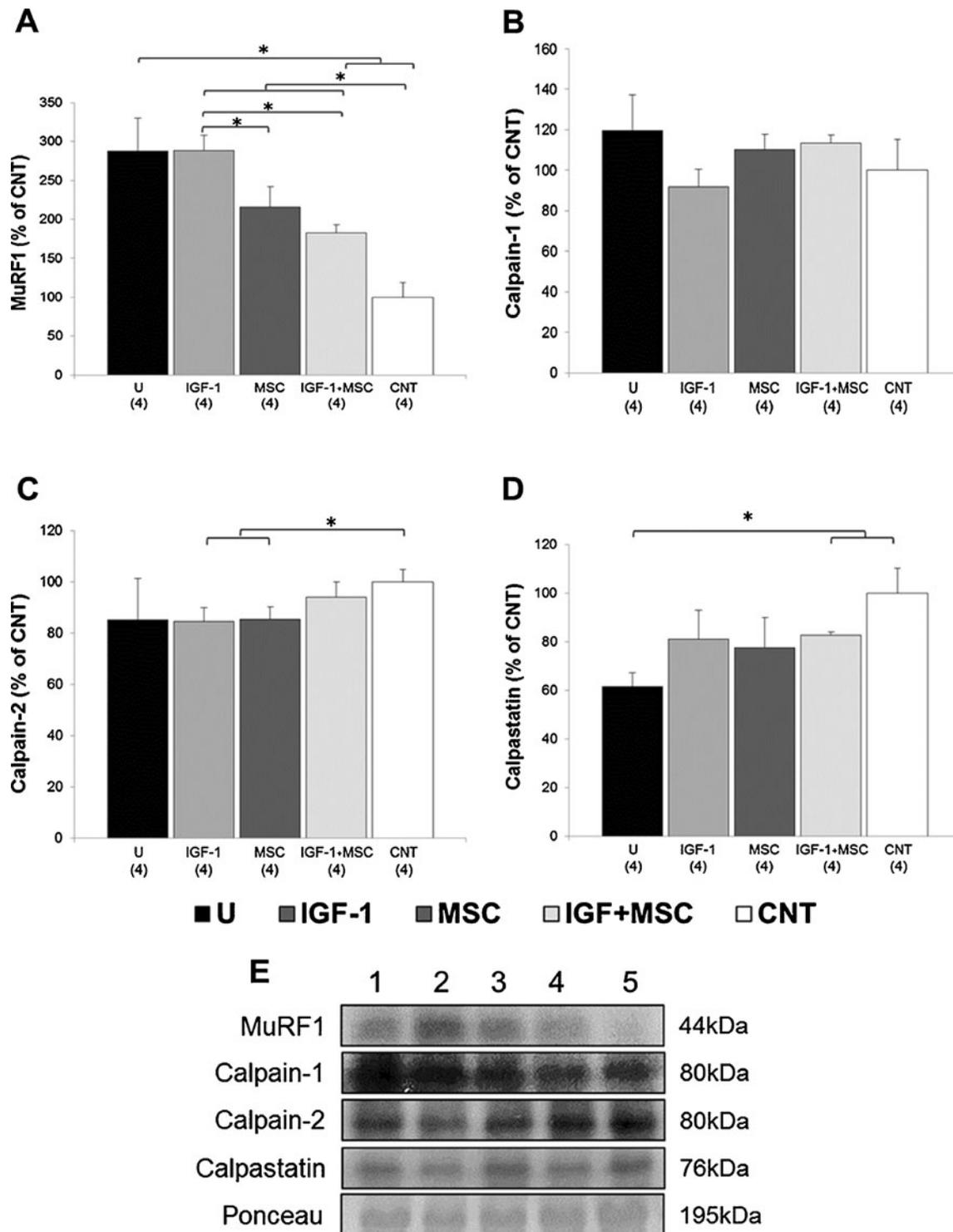
**Fig. 6** Association of both MSCs and IGF-1 modulates inflammation through reduction of inflammatory molecules in skeletal muscle of LAMA2<sup>dy/2j</sup> dystrophic model. **a** Western Blot analysis of TNF- $\alpha$  expression in muscle samples from untreated dystrophic mice (U), IGF-1 treated mice (IGF-1), MSC treated mice (MSC), both IGF-1 and MSC treated mice (IGF-1 + MSC), in comparison with non-dystrophic mice (control; CNT) expression. Data correspond to optical density of respective protein bands, assessed by ImageJ software; **b** Representative Western

Blot bands. *Samples:* 1) Untreated dystrophic mice; 2) IGF-1 treated mice; 3) MSC treated mice; 4) IGF-1 + MSC treated mice; 5) Non-dystrophic mice (CNT); **c-g** Real-time Quantitative RT-PCR analysis of CCL2, CCL8, CCL5 and CCL3 expression in muscle samples of untreated and treated mice, compared with control. The number of animals in each group is shown in *parentheses* and data are presented as mean  $\pm$  SE. \* $P < 0.05$ ; Student's *t* test



**Fig. 7** Association of both MSCs and IGF-1 contributes to regeneration process in *Lama2<sup>dy2j</sup>* dystrophic muscle. **a,b** Western Blot analysis of Myogenin and MyoD expression in muscle samples from untreated dystrophic mice (U), IGF-1 treated mice (IGF-1), MSC treated mice (MSC), both IGF-1 and MSC treated mice (IGF-1 + MSC), in comparison with non-dystrophic mice (control; CNT) expression. Data correspond to optical density of respective protein bands, assessed by ImageJ

software; **c** Representative Western Blot bands. *Samples*: 1) Untreated dystrophic mice; 2) IGF-1 treated mice; 3) MSC treated mice; 4) IGF-1 + MSC treated mice; 5) Non-dystrophic mice (CNT); **d-f** Real-time Quantitative RT-PCR analysis of Myf5, MyoD and Myogenin expression in muscle samples of untreated and treated mice, compared with control. The number of animals in each group is shown in *parentheses* and data are presented as mean  $\pm$  SE. \* $P < 0.05$ ; Student's *t* test



**Fig. 8** Association of both MSCs and IGF-1 affects expression of proteins related with proteolytic systems in muscle of LAMA2<sup>dy/2j</sup> dystrophic model. **a-d** Western Blot analysis of MuRF1, Calpain-1, Calpain-2, and Calpastatin in muscle samples from untreated dystrophic mice (U), IGF-1 treated mice (IGF-1), MSC treated mice (MSC), both IGF-1 and MSC treated mice (IGF-1 + MSC), in comparison with non-dystrophic mice (control; CNT) expression. Data correspond to

optical density of respective protein bands, assessed by ImageJ software; **e** Representative Western Blot bands. *Samples:* 1) untreated dystrophic mice; 2) IGF-1 treated mice; 3) MSC treated mice; 4) IGF-1 + MSC treated mice; 5) Non-dystrophic mice (CNT). The number of animals in each group is shown in *parentheses* and data are presented as mean  $\pm$  SE. \* $P < 0.05$ ; Student's *t* test

myogenic differentiation of injected MSCs. These results may have important applications for future therapy in patients with different forms of muscular dystrophies.

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**Disclosures** The authors indicate no potential conflicts of interest.

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